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BRUTON TYROSINE KINASE IN IMMUNODEFICIENCY AND IN B-CELL MALIGNANCY

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Bruton Tyrosine Kinase in Immunodeficiency and in B-Cell Malignancy

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To my family

ABSTRACT

BTK inhibitors have induced high response rates in the treatment of leukemias and lymphomas. Ibrutinib is the first-in-class US Food and Drug Administration (FDA)-approved BTK covalent inhibitor to treat chronic lymphocytic leukemia (CLL). However, a sub-group of patients develops resistance to ibrutinib therapy. The most common resistance mechanism is substitution of cysteine 481 to serine of BTK, the residue to which ibrutinib binds irreversibly. Few other amino acid replacements at this site had been characterized. In paper I, we therefore performed functional analysis of all the possible amino acid replacements at C481 site due to a single nucleotide change. We also included threonine substitution because of its structural similarity to serine. BTK with cysteine-to-threonine substitution retains catalytic activity and cause ibrutinib resistance. Thus, we identified a new potential resistant variant, BTK C481T for BTK irreversible inhibitors. For the replacement with arginine, phenylalanine, tryptophan or tyrosine, BTK enzymatic activity was completely ablated, while glycine substitution still showed some kinase activity, but to a much lower extent.

CLL patients receiving ibrutinib treatment show rapid mobilization of tumor cells from lymph nodes (LN) to peripheral blood (PB). However, the detailed mechanism of ibrutinib-induced tumor cell redistribution has not been clarified. In paper II, we tried to explore this observation by analyzing changes in plasma inflammation-related biomarkers, transcriptional levels in CLL cells, B-cell activation and migration markers, and PB mononuclear cell populations as early as 9h after ibrutinib treatment. We compared the changes between before and at 6 time points after treatment initiation in LN and PB. We observed a significant downregulation of 10 plasma inflammation-related markers, mainly chemokines but not CLL-derived within 9 hours. RNA-sequencing data showed significant and continuous expression changes of genes related to B-cell receptor (BCR) signaling and CLL biology. We conclude that ibrutinib rapidly blocks an ongoing inflammatory response and in particular influences LN CLL cells.

Loss-of-function (LOF) mutations of BTK lead to a severe block in B lineage development, as seen in X-linked agammaglobulinemia (XLA). In paper III, we analyzed a large number of XLA patients, including 108 previously unreported cases. The tolerance to single amino acid replacements was predicted for full-length BTK. Moreover, we compared these germline XLA-causing mutations with those acquired in leukemia and lymphoma. Based on published cases and those reported to a mutation database, BTK mutation spectrum in more than 10,000 BTK-independent tumors was compared to the BTK-dependent malignancies, CLL and mantle cell lymphoma (MCL), and also to BTK-potentially-dependent malignancies, like diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and germinal center (GC)-derived B-cell lymphoma. This analysis for the first time identifies BTK to be a potential tumor suppressor in a subset of DLBCL and FL. Therefore, whether BTK inhibitors, which show highly efficient therapeutic effects in CLL and MCL, might promote tumorigenesis in a subset of other B cell malignancies remains an open question.

LIST OF SCIENTIFIC PAPERS

I. Hamasy A*, **Wang Q***, Blomberg KE, Mohammad DK, Yu L, Vihinen M, Berglöf A, Smith CI. **Substitution scanning identifies a novel, catalytically active ibrutinib-resistant BTK cysteine 481 to threonine (C481T) variant.** *Leukemia.* 2017 Jan;31(1):177-185.

II. Palma M*, Krstic A*, Peña Perez L, Berglöf A, Meinke S, **Wang Q**, Blomberg KEM, Kamali-Moghaddam M, Shen Q, Jaremko G, Lundin J, De Paepe A, Höglund P, Kimby E, Österborg A, Månsson R, Smith CIE. **Ibrutinib induces rapid down-regulation of inflammatory markers and altered transcription of chronic lymphocytic leukaemia-related genes in blood and lymph nodes.** *Br J Haematol.* 2018 Oct;183(2):212-224.

III. **Qing Wang**, Anna Berglöf, A. Charlotta Asplund, Rula Zain, Igor Resnick, Hernando Yesid Estupiñan Velasquez, Sofia Khan, Gerard C.P. Schaafsma, Mauno Vihinen, and C. I. Edvard Smith. **Acquired BTK variations suggest tumor suppressor activity in leukemia and lymphoma subsets.** (Manuscript)

* These authors contributed equally.

Additional scientific papers that are not included in the thesis.

I. Lundin KE, **Wang Q**, Hamasy A, Marits P, Uzunel M, Wirta V, Wikström AC, Fasth A, Ekwall O, Smith CIE. **Eleven percent intact PGM3 in a severely immunodeficient patient with a novel splice-site mutation, a case report.** *BMC Pediatr.* 2018 Aug 29;18(1):285.

II. Gustafsson MO, Mohammad DK, Ylösmäki E, Choi H, Shrestha S, **Wang Q**, Nore BF, Saksela K, Smith CI. **ANKRD54 preferentially selects Bruton's Tyrosine Kinase (BTK) from a Human Src-Homology 3 (SH3) domain library.** *PLoS One.* 2017 Apr 3;12(4):e0174909.

III. Bestas B, Turunen JJ, Blomberg KE, **Wang Q**, Månsson R, El Andaloussi S, Berglöf A, Smith CI. **Splice-correction strategies for treatment of X-linked agammaglobulinemia.** *Curr Allergy Asthma Rep.* 2015 Mar;15(3):510.

IV. Hernando Yesid Estupiñan Velasquez, Yuye Shi, Dara K. Mohammad, **Qing Wang**, Liang Yu, Mauno Vihinen, Rula Zain, Anna Berglöf, Edvard CI Smith. **Combined gatekeeper and C481-substitutions lead to super resistance for irreversible BTK inhibitors acalabrutinib, ibrutinib and zanubrutinib.** (Manuscript)

CONTENTS

LIST OF ABBREVIATIONS

ALC	Absolute lymphocyte count
ANKRD54	Ankyrin repeat domain 54
AP-1	Activator protein 1
APRIL	A proliferation-inducing ligand
ARA	Autosomal recessive agammaglobulinemia
BAFF	B-cell activating factor
BAG6	BCL2 associated athanogene 6
BCL-2	B-cell lymphoma 2
BCR	B-cell receptor
BLK	B-lymphocyte kinase
BLNK	B-cell linker protein
BMSC	Bone marrow stromal cell
BMX	Bone marrow tyrosine kinase gene in chromosome X protein
Bright	B-cell regulator of Ig heavy chain transcription
BTK	Bruton tyrosine kinase
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CLL	Chronic lymphocytic leukemia
CSK	C-terminal Src kinase
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCL13	Chemokine (C-X-C motif) ligand 13
CXCR4	C-X-C chemokine receptor 4
CXCR5	C-X-C chemokine receptor 4
DCs	Dendritic cells
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein–Barr virus
EGFR	Epidermal growth factor receptor

FDA	US Food and Drug Administration
GVHD	Graft versus host disease
GC	Germinal center
HCK	Hematopoietic cell kinase
HER	Human epidermal growth factor receptor
HSCs	Hematopoietic stem cells
Ig	Immunoglobulin
IGHM	Immunoglobulin Heavy Constant Mu
IL-10	Interleukin-10
IP	Immunoprecipitation
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITK	IL2-inducible T-cell kinase
JAK3	Janus kinase 3
KD	Kinase domain
LCK	Leukocyte C-terminal Src kinase
LN	Lymph node
LOF	Loss-of-function
LYN	LCK/YES novel tyrosine kinase
M-CLL	IgV-mutated CLL
MCL	Mantle cell lymphoma
MYD88	Myeloid differentiation primary response 88
MZL	Marginal zone lymphoma
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NLC	Nurse like cell
OS	Overall survival
ORR	Overall response rate
PB	Periphery blood

PBMC	Peripheral blood mononuclear cell
pDCs	Plasmacytoid dendritic cells
PEA	Proximity extension assay
PEI	Polyethylenimine
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIDs	Primary immunodeficiency diseases
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PLCγ2	Phospholipase C gamma 2
PTKs	Protein tyrosine kinases
pY	Phosphotyrosine
RA	Rheumatoid arthritis
RLK	Resting lymphocyte kinase
R/R	Relapsed and/or refractory
RT	Room temperature
SAS	Solvent-accessible surface
SH2	Src homology 2
SH3	Src homology 3
SYK	Spleen tyrosine kinase
TFKs	TEC family kinases
TGF-α	Transforming growth factor alpha
TH	Tec homology
TNF-β	Tumor necrosis factor beta
TME	Tumor microenvironment
Treg	T regulatory cells
UM-CLL	IgV-unmutated CLL

WM	Waldenström macroglobulinemia
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

1 INTRODUCTION

1.1 PRIMARY IMMUNODEFICIENCY DISEASES (PIDS)

The group of primary immunodeficiency diseases (PIDs) contains numerous genetic disorders that influence various components of the innate and adaptive responses[1]. The first description of a PID with information about the underlying mechanism was reported in 1952 when X-linked agammaglobulinemia (XLA) was discovered by Ogden Bruton[2], which will be discussed in detail in the next section. After the discovery of XLA, more than 350 unique immunodeficiency disorders have been characterized. Primary B-cell deficiencies account for two-thirds of PIDs, and the most severe form, which leads to reduction of immunoglobulins (Igs) of all isotypes is called agammaglobulinemia. There are two major types of agammaglobulinemia, XLA and autosomal recessive agammaglobulinemia (ARA).

In the majority of patients with PIDs, the most common observation is deficiency in the antibody production[3]. The characteristic of both XLA and ARA is increased susceptibility to bacterial infections, the most prevalent being respiratory and/or gastrointestinal tract infections. The infection occurs often in the first year of life after the disappearance of the maternal IgG[4]. In both XLA and ARA, B-lymphocytes and plasma cells are dramatically reduced, but the number and phenotype of other cell lineages are usually normal. It is highly suggestive that the differentiation disorder is only confined to the B-cell lineage. This characterization distinguishes XLA and ARA from many other antibody deficiencies, which not only have the defect of B-lymphocytes, but also are often accompanied by T-lymphocyte deficiencies and autoimmunity[5–7].

Currently, the treatment for PID patients is intravenous and subcutaneous administration of immunoglobulin and antibiotic therapies. The human agammaglobulinemia with a known defective genetic component is summarized in Table 1. Since the characteristics of both XLA and ARA are similar, the investigation of the gene mutations would facilitate diagnosing diseases and also explore appropriate treatments for patients.

Table 1. Human agammaglobulinemia with a known defective genetic component.

GENE SYMBOL	PROTEIN	INHERIANCE
BTK[8]	Bruton tyrosine kinase	XLA
BLNK[9,10]	B-cell linker protein	ARA
CD79A[9,11]	B-cell antigen receptor complex-associated protein alpha	ARA
CD79B[12,13]	B-cell antigen receptor complex-associated protein beta	ARA
IGHM[9,14–16]	Immunoglobulin Heavy Constant Mu	ARA
IGLL1[9,17]	Immunoglobulin lambda-like polypeptide 1	ARA
PIK3R1[18]	Phosphatidylinositol 3-kinase regulatory subunit alpha isoform 1	ARA

In conclusion, the mutations of genes coding for the pre-B cell receptor (pre-BCR)/BCR or downstream molecules related with these receptors cause agammaglobulinemia in humans (Figure 2)[4]. However, the genes summarized in Table 1 are at normal status in many patients with agammaglobulinemia. Considering the large number of downstream components associated with pre-BCR/BCR, new mutations are expected to be identified among the downstream molecules. It seems to be reasonable to propose that mutations in genes coding for further downstream molecules of the pre-BCR/BCR will not cause 'pure' agammaglobulinemia, since they are also frequently expressed in other cell types involved in different signaling pathways[4].

1.1.1 B-lymphocyte development

In humans, B-cell development is a highly regulated procedure, which starts from hematopoietic stem cells (HSCs) in the bone marrow. There are several stages during the development, pro-B cell, pre-B cell, immature B-cell and mature B-cell. The first section of B-lymphocyte development occurring in the bone marrow, includes pro-B cell to immature B-cell stages. These immature cells can be easily eliminated upon interaction with self-antigens. Immature B-lymphocytes express high IgM and low IgD (IgM^{high}/IgD^{low}). When the cells exit from the bone marrow and enter to the peripheral environment, the immature B-cells become mature and express IgG, low IgM and high IgD (IgM^{low}/IgD^{high})[19,20]. Mature B-cells can migrate to the second lymphoid tissues, where they can differentiate into memory B-lymphocytes or plasma cells[21].

In the bone marrow, at the early stage of B-cell development, there is a unique mechanism of genetic rearrangement called V(D)J recombination. This procedure rearranges variable (V), diversity (D) and joining (J) gene regions, which eventually results in the antigen-binding

segments of Igs. Human antibodies are composed of heavy chains and light chains, which harbor both constant (C) and variable (V) regions. In the B-cell development, the first recombination event is D-J recombination and then followed by linking to V gene segment, which forms a rearranged VDJ gene complex ultimately resulting in the production of the IgM heavy chain. The recombination is similar in the light chain, except lacking a D segment. The assembly of the heavy chain with one of the light chains leads to the formation of IgM that can be found on both immature and mature B-cells[22].

In mature B-cells, antigen binding to BCR induces a series of signaling events that ultimately activate some transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1). These transcription factors can regulate expression of the genes which are related with B-cell survival, differentiation and activation[23–25]. However, BCR can still transduce a constitutive signaling in a low level even without antigen binding and this process is called tonic signaling. Tonic signaling is also required for B-cell survival, which has been illustrated by the finding that ablation of BCR expression *in vivo* in mice induces rapid B-cell apoptosis[26].

1.1.2 X-linked agammaglobulinemia (XLA)

As described above, XLA is a hereditary PID due to mutations of the Bruton tyrosine kinase (*BTK*) gene, which encodes a nonreceptor tyrosine kinase[27]. The *BTK* gene localizes to the short arm of the X chromosome in the 21.3-22 region (Xq21.3-Xq22)[28–30]. The structure and expression of the BTK protein will be discussed in details later. In XLA patients, the numbers of B-lymphocytes are dramatically decreased due to the block of B-cell development, which leads to low serum Igs of all isotypes[31,32]. As a consequence, patients with XLA are very susceptible to bacterial and enteroviral infections and the most typical infections are from *Haemophilus influenza* and *Streptococcus pneumonia*[33,34].

XLA essentially only occurs in males whereas females are usually healthy even carrying XLA-causing BTK mutations. This is because the non-random X chromosome inactivation cells that express normal BTK have selective advantage[35]. The incidence for XLA has been estimated to be 1:200,000. There is a high appearance of this X-linked disease in males and they are easily diagnosed at molecular and clinical levels. The corresponding spontaneous disease for XLA in mice is called X-linked immunodeficiency (Xid) and it is much milder than XLA[36]. Xid in mice is caused by the cysteine substitution at the Arg28 site in the PH domain[37], which is one of the most frequently mutated amino acids in BTK.

1.1.3 BTKbase

The human proteome contains around 500 protein kinases. Among these protein kinases, BTK has the largest number of disease-causing mutations[38]. A big amount of mutations in

BTK can induce XLA, which includes amino acid substitution, nonsense mutation, frameshift mutation caused by insertion or deletion, and splice-site mutation. XLA-causing mutations are compiled in a database called BTKbase, which was established in 1994 and this online resource is being updated continuously[39–41]. In 2005, our group published a review summarized the mutations. At that time there were 554 unique molecular events included in the BTKbase[42]. Along with the data updating, a total number of 1800 of public variants representing 920 unique public DNA mutants have been recorded in the BTKbase currently[41]. For each patient, the following information (if available) is incorporated to the database: the identification of the entry, a detailed characterization of the mutation with the corresponding reference, explanation of the mutation at different levels, and distinct parameters from patients[8].

XLA-causing mutations have been reported in all the domains and also in the noncoding regions of the *BTK* gene. All types of mutations are distributed throughout the whole BTK. Among all the reported XLA-causing mutations, amino acid substitutions are predominant, and account for two-thirds of all mutations. Amino acid replacements are scattered all over the whole BTK except for the Src homology 3 (SH3) domain. So far, only two amino acid substitutions in this domain have been found to cause XLA[41,43]. Amino acid substitutions appear in the kinase domain (KD) with the highest frequency, which is probably because the KD contains several highly conserved regions that are important for the BTK catalytic activity[44].

Among all the amino acids, arginine is the most frequently mutated amino acid due to that some arginine codons contain the CpG-doublet[45]. CpG dinucleotides are mutational hotspots and are found in four out of the six codons for arginine. Thereby, arginine is the most often replaced residue by other amino acids. Mutations of arginine can also give rise to a stop codon, by a CGA to TGA change causing XLA due to the production of an unstable, missing protein. Totally, there are four such CGA codons in BTK, Arg13, Arg255, Arg520 and Arg525[42]. At these four positions, nucleotide substitutions could also generate stop codon leading to XLA. However, the mutation scenarios are different among the four sites. So far, at residues Arg13 and Arg255, only stop codons have been reported to cause XLA, whereas no XLA-causing missense mutations have been identified. Comparing with Arg520 and Arg525, it is highly suggestive that amino acid replacements could be tolerated at Arg13 and Arg255 ($P<0.001$)[42]. It is well known that arginine is the residue with the highest mutation frequency because of the mutational hotspot. Arginine may also be difficult to replace due to its elongated and charged side chains having many functional and structural interactions[45]. On the other hand, substitution with proline is found to be the most common XLA-causing replacement[42], presumably because this substitution frequently disturbs protein conformation, thereby altering both stability and function of BTK.

In addition, amino acid substitutions occurring in secondary structural elements are more likely to cause disease than those found in loop regions. This phenomenon is probably due to the higher flexibility in loops than in helices and strands. In the wild-type BTK, the relative

solvent-accessible surface (SAS) value is a good indicator for the solvent accessibility at each site[44].Therefore, residues could be classified into three categories: buried, exposed and intermediate[44]. This classification facilitates predicating the pathogenicity of amino acid substitutions. In general, the majority of buried sites are highly conserved whereas most exposed residues are weakly conserved. Variations at conserved and buried residues are the most frequently disease-causing because substitutions at these sites are prone to affect protein stability. Similarly, those small amounts of conserved exposed residues are associated with pathogenicity strongly[44].

One of the initial purposes of BTKbase was to study the mutation characteristics and try to build up the genotype–phenotype correlations[8]. Although this connection has not been demonstrated, it should be kept in mind that almost all the identified patients manifest the classical severe pattern of the disease with only a few exceptions. For example, as for amino acid substitution, replacements by related amino acids are expected to cause a milder form of disease. Therefore, the definitive genotype–phenotype relationship was predicted to be exist, but further investigation is needed[42]. In addition, the mutation types might influence the disease severity such as the mutations in less conserved regions e.g., amino acid replacements in non-invariant positions or splicing defects, which might still induce partial production of BTK and cause milder XLA[9,42].

So far, a big amount of BTK mutations have been found to cause XLA and are recorded in the BTKbase. Some novel mutations would also show up in the future. Therefore, it is really crucial to maintain the BTKbase, which collects all the known mutations and will be continuously updated along with the increasing exploration of this field. The BTKbase has been applied in different studies, such as phenotype-genotype correlations as discussed above and the prediction of the pathogenicity, which facilitates analyzing and diagnosing diseases, and developing the precise medicines as well[44].

1.2 PROTEIN TYROSINE KINASES (PTKS)

1.2.1 The TEC family of tyrosine kinases

The TEC family kinases (TFKs) are the second-largest group of nonreceptor PTKs. They are critical components in several signal transduction pathways that mediate cell development, differentiation, proliferation and apoptosis. The TFKs consist of five members; BTK, TEC, IL2-inducible T-cell kinase (ITK), resting lymphocyte kinase (RLK/TXK) and bone marrow tyrosine kinase gene in chromosome X protein (BMX)[46].

The expression pattern among the TFKs varies, but all except for BMX are primarily appearing in the hematopoietic cells[27,46–52]. BTK is constitutively expressed in all hematopoietic cells except for T-lymphocytes and plasma cells[47]. Tec is expressed in B- and T- cells, myeloid cells, and endothelial cells[53,54]. ITK is the highest expressed TFKs member in T-cells and also exists in natural killer (NK) cells and mast cells[49,55]. ITK deficiency in

humans causes susceptibility to serious, often lethal, Epstein–Barr virus (EBV) infection[56]. RLK/TXK has been reported to be expressed in the T-lymphocytes, preferentially in Th1 cells, and NK cells[51,55]. BMX was initially identified in the bone marrow but mainly seems to exist in endothelial cells[48]. In some lineages, more than one TFK member is expressed. For instance, both BTK and TEC are found in B-cells, whereas ITK and RLK/TXK express in T-cells. Moreover, these kinases are also found to be expressed in other species, such as *Drosophila melanogaster*[57] and zebra fish[58].

The structure of all the five TFK members is similar. It consists of pleckstrin homology (PH) domain at the N-terminus, followed by Tec homology (TH), SH3, SH2 domains and KD. However, the structure of RLK/TXK is an exception with a unique region containing a palmitoylated cysteine string in the N terminus[59] (Figure 1).

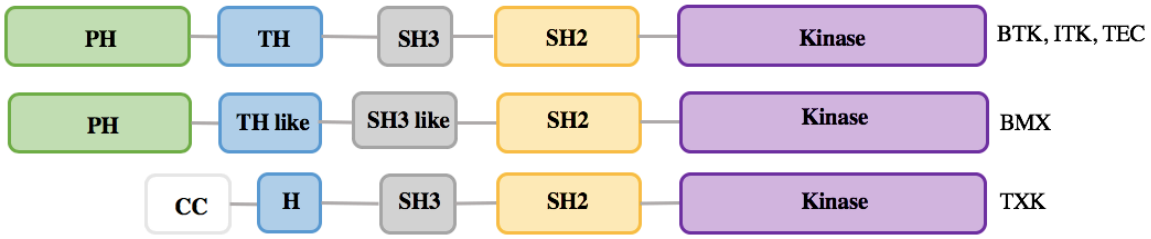


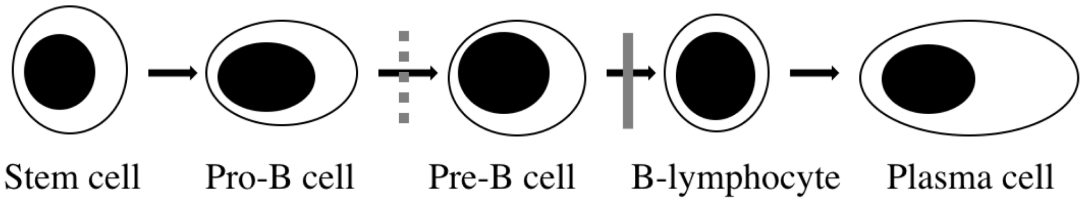
Figure 1. Schematic diagram of the domain structure of TFK members (modified from[46]).

The N-terminal PH domain mediates binding to the membrane, whereas, in TXK, the palmitoylated cysteine string has a similar function as the PH domain. The TH domain consists of a highly conserved zinc binding BTK motif[60,61] and followed by a Proline-rich region. In BMX, the residues surrounding the Proline-rich region are not conserved as in other TFKs, thus it is referred as a "TH like" domain[46]. The SH3 domain is involved in managing protein-protein interactions which are crucial for various signal transduction processes[62]. Similar with the SH3 domain, the SH2 domain also regulates protein interaction but involves in myriad phosphotyrosine (pY)-signaling pathways[63]. Unlike other members, the SH3 domain in BMX is truncated, therefore, it is called "SH3 like" domain[46]. The kinase domain at the C-terminus is essential for the kinase activity in all TFK members.

1.2.2 BTK

As described in the previous chapter, BTK belongs to the TFKs and it is a crucial molecule in B-cell development, differentiation and proliferation. BTK consists of 659 amino acids and its molecular weight is 77 kDa. BTK expression starts from early stage of B-cell development and it can be found among all the stages before plasma cells[47,64,65]. In XLA, BTK mutations cause a partial block at the pro- to the pre-B cell stage, as well as, a completely developmental block at the mature B-lymphocyte stage (Figure 2A)[31,32]. Correspondingly, in Xid mice, there is a later and only partial B-cell developmental block between the pre-B and mature B-cell stage (Figure 2B)[36,66].

A.



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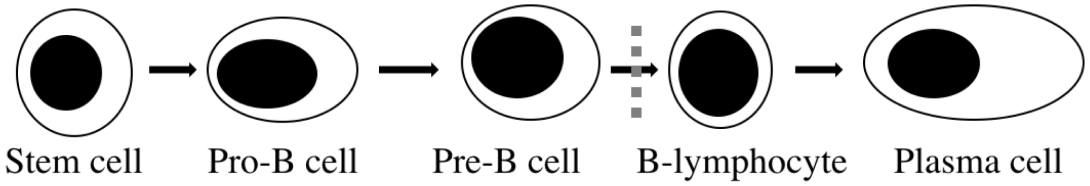

Figure 2. A schematic representation of B-lymphocyte blockage in XLA (A) and in Xid (B).

It is well known that BTK is a cytoplasmic non-receptor tyrosine kinase. However, BTK can also be detected in the nucleus[67,68]. Moreover, it is reported that the nucleocytoplasmic shuttling of BTK is mediated by the interaction with ankyrin repeat domain 54 (ANKRD54) and the interaction is SH3 dependent[69]. A paper published by our group suggested that the nucleus exit of both BTK and another TFK, TXK is mediated by ANKRD54 and it depends on the SH3 domain entirely[69]. In the cytoplasm, BTK plays a pivotal role in the BCR signaling pathway, whereas, the role of BTK in the nucleus is poorly understood. It has been demonstrated by one group of researchers that the DNA binding of a B-cell transcription factor, Bright (B-cell regulator of Ig heavy chain transcription), was dependent on the presence of BTK[67].

BTK is a crucial component involved in BCR signaling pathway, which regulates survival and proliferation of both normal cells and malignant cells. This will be discussed in details in the next chapters. However, few reports also proposed that BTK could function as a tumor suppressor[70–72]. It was found that BTK could act as a tumor suppressor when cooperated with a homozygous loss-of-function (LOF) of the B-cell linker protein BLNK/SLP-65 in pre-B cells and this was independent of BTK catalytic activity in the animal model[70,71]. In addition, there have also been a few literatures from patients suggesting an influence of the *BTK* gene[72–74]. For example, Rada, M., *et al* have proposed that BTK functions as a tumor suppressor via stabilizing p53 protein expression[72]. The p53 protein is one of the most potent tumor suppressors and it could increase the BTK expression. This suggests that the relationship between BTK and p53 is regulated by a positive feedback loop, with the eventual goal of strengthening p53 activity upon damage responses[72].

1.2.3 B-cell receptor (BCR) pathway

As described previously, BCR signaling is essential for B-cell differentiation, proliferation and apoptosis. The BCR is a transmembrane receptor protein, which is constituted by two identical IgL chains, two identical IgH chains and the heterodimer co-receptor Igα (CD79A) and Igβ (CD79B)[75,76]. Both Igα and Igβ contain the immunoreceptor tyrosine-based activation motifs (ITAMs) at the cytoplasmic tail, which play a crucial role in signal transduction[75,77].

Upon B-cell receptor activation, the tyrosine residues in ITAMs are phosphorylated by the SRC-family protein tyrosine kinase such as LCK/YES novel tyrosine kinase (LYN), which create docking sites for spleen tyrosine kinase (SYK)[78]. BTK translocates to the plasma membrane via interactions between its PH domain and the phosphatidylinositol-3,4,5-triphosphate (PIP3) which is generated by phosphatidylinositol-3-kinase (PI3K)[79]. Meanwhile, the SRC-family kinases, in particular LYN and SYK are activated, resulting in transphosphorylation of Y551 in BTK which promotes the BTK enzymatic activity and subsequently auto-phosphorylates the Y223 in the SH3 domain[80,81]. Accordingly, PLCγ2 is phosphorylated and activated[82]. A signalosome of multiple protein tyrosine kinases and adaptor proteins consisting of SYK, BTK, BLNK and PLCγ2 is formed and results in the calcium mobilization and protein kinase C (PKC) activation[24,83–85]. This is followed by the activation of various transcription factors, which regulate B cell survival or apoptosis, proliferation, and differentiation[86].

The BCR is critical for normal B-cell development and maintenance. Meanwhile, BCR signaling is also a pivotal pathway that promotes progression of B-cell malignancies[87,88]. Therefore, the development of specific inhibitors targeting components involved in the BCR signaling pathway holds promising to treat B-cell malignancies, which we will discuss in the following chapters.

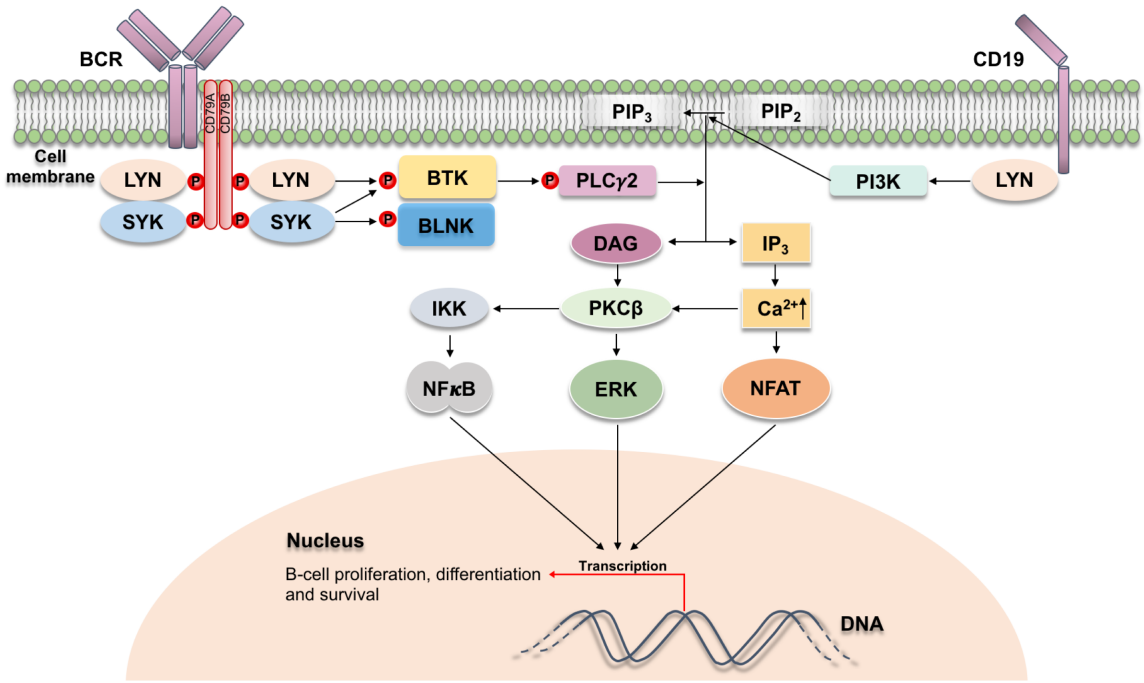

Figure 3. The schematic representation of BCR signaling pathways (modified from[86]).

1.3 B CELL MALIGNANCIES

1.3.1 Chronic lymphocytic leukemia (CLL)

CLL is a type of chronic lymphoid malignancy for which the growth of B-lymphocytes is out of control and cells are resistant to apoptosis. In CLL, $CD5^{+}CD23^{+}$ monoclonal B-cells are accumulated in primary and secondary lymphoid organs. CLL mainly occurs in elderly people, with a median age of over 70-years at the time of diagnosis and the incidence in males is twice as in females[89].

The symptoms, and physical and laboratory findings in CLL patients are various at the time of diagnosis. Some patients are diagnosed as having CLL due to the enlargement of lymph nodes. It usually occurs in the cervical area, where the swelling decreases and increases spontaneously but does not disappear completely. Based on a high white blood cell count, particularly a large accumulation of circulating lymphocytes by a regular blood test, many patients have no symptoms when diagnosed with CLL[89,90].

In general, CLL is originated from autoreactive germinal center (GC) B-lymphocytes with constitutive BCR activation[91–94]. BCR is crucial for the proliferation and survival of normal B lymphocytes as mentioned before. Most B-cell lymphoproliferative disorders have functional surface BCR expression, including CLL. Depending on the mutational condition of the BCR, CLL can be classified into two types, heavy-chain variable region gene-unmutated (IgV-unmutated) CLL (UM-CLL) and IgV-mutated CLL (M-CLL)[91,95]. It has been suggested that the M-CLL originates from antigen-experienced B-lymphocytes which have went through the GC of secondary lymphoid tissues, where the Ig somatic hypermutation occurs[96]. However,

whether UM-CLL is derived from GC-independent antigen-experienced B-lymphocytes or naïve B-cells is still unknown[96]. The gene expression profiling between CLL samples and subpopulations of untransformed human B-cells was compared, which showed that both UM-CLL and M-CLL cells had similar features with $CD27^+$ memory B-lymphocytes[96]. This suggests that these two CLL subtypes are derived from antigen-experienced $CD27^+$ memory B-lymphocytes, but UM-CLL and M-CLL originate from GC-independent or post-GC cells, respectively[96,97]. However, other report has suggested that UM-CLL is derived from $CD5^+CD27^-$ naive B-cells whereas M-CLL resembles $CD5^+CD27^+$ post-GC subset[98]. In general, the suggestion about M-CLL presumably originates from GC-experienced B-lymphocytes whereas UM-CLL derives from pre-GC-naive B-lymphocytes or GC-independent memory B-lymphocytes still remains on debate[99].

The disease is more aggressive in patients with UM-CLL than patients with M-CLL[100]. Previous studies reported that M-CLL patients experienced longer overall survival (OS) than UM-CLL patients[100,101]. Patients with M-CLL had a median OS of over 20 years while those with UM-CLL only had median OS of 8 years. Moreover, other gene mutations such as *TP53* resulting in p53 inactivation, is also a high risk factor in CLL patients leading to shorter OS[102–104].

The initial treatments for CLL patients vary depending on the situation of patients, such as the exact diagnosis and the progression of the disease. The treatments include chemotherapy, bone marrow transplantation, radiation therapy or biological therapy. However, there is no disease progression in a small group of patients throughout their lifetime and therefore therapy is not required for them. But a substantial group of patients will eventually need treatment due to active CLL[105].

In this thesis, we concentrated on the biological therapy, which also called chemoimmunotherapy. Before treatment, some prognostic molecular markers are required to be identified in CLL patients, which are useful for assessing the individual risk of CLL progression. This information can provide recommendations for designing appropriate individualized therapy programs. The risk factors contain mutational status of *TP53* and the BCR *IGHV* genes as discussed above, cytogenetic abnormalities for instance del(11q), del(13q), del(17p), and trisomy 12. CLL patients harbor high-risk molecular and/or cytogenetic features, which are those with *TP53* disruption, UM-CLL, del(11q), or del(17p), and correspondingly, low-risk patients have M-CLL, del(13q) and trisomy 12.

In treatment naïve CLL patients who require therapy, the presence of del17p and *TP53* disruptions should be tested is a general consensus[99]. Patients with mutated *TP53* and/or del17p are mostly recommended to be given the BTK inhibitor, ibrutinib, as the upfront treatment[106]. Whereas, for those patients who are not suitable for ibrutinib treatment because of some comorbidities or drug interactions, the B-cell lymphoma 2 (BCL-2) inhibitor, venetoclax is an excellent option[107]. In patients with wild-type *TP53*, the *IGHV* mutational status and physical conditions need to be considered for individualized treatment. In the high-risk group of patients with UM-CLL, regardless of comorbidities and age, ibrutinib therapy in

the first-line setting is a growing consensus[99]. However, the treatment for low-risk CLL will still be on debate until more data are obtained from ongoing randomized clinical tests[99].

1.3.2 BTK inhibitors

1.3.2.1 First generation

1.3.2.1.1 Ibrutinib

Kinase inhibitors have a huge impact on cancer therapy, and inhibitors for BTK have revolutionized the treatment of leukemias and lymphomas over the last few years[108]. Ibrutinib is the first approved BTK inhibitor by FDA to treat several malignancies. The generation of ibrutinib originated from a series of small molecule inhibitors of BTK, which were synthesized in 2007 by Pan, Z., *et al*[109]. Ibrutinib, also called PCI-32765, was selected from many BTK inhibitors for further clinical trials because it showed high specificity and efficacy for inhibiting BTK activity in experimental models[109]. This compound exhibited increasingly clinical activity in several B-cell lymphomas and has been FDA-approved to treat malignancies, including CLL[110], mantle cell lymphoma (MCL)[111], marginal zone lymphoma (MZL)[112], Waldenström macroglobulinemia (WM)[113] and graft-versus-host disease[114]. Currently, ibrutinib is the only approved BTK inhibitor for CLL treatment, in both the front-line and relapsed and/or refractory (R/R) disease conditions.

Ibrutinib binds to a conserved cysteine residue (Cys481) at the ATP binding site of BTK covalently and irreversibly, and thus block BCR signal transduction[115–117]. As mentioned above, in addition to be approved to treat several B-cell lymphomas, ibrutinib also showed encouraging preclinical efficacy to apply on autoimmune and inflammatory disorders[118,119].

After oral administration, ibrutinib is absorbed rapidly and reach the maximum serum concentration, around 0.4 μM in one to two hours[110]. Since ibrutinib binds to BTK covalently and irreversibly, it can be given to patients once daily with the dose of 420mg even though the half-life of this compound is short (4-6 hours)[110,120].

As described above, ibrutinib is the first FDA-approved BTK inhibitor to treat CLL. However, with the long-term follow-up investigation, it has shown that up to 51% of patients have suspended ibrutinib treatment due to its intolerance and complications[121–124]. The common adverse effects of ibrutinib therapy are diarrhea, bleeding events, infections, arthralgia, dermatologic events, and hypertension[121,125–129]. There are also some additional rare side effects including atrial fibrillation, pneumonitis, neutropenia and major hemorrhage.

These adverse effects could arise from off-target binding of ibrutinib because it has a potential to inhibit activity of other tyrosine kinases which contain similar cysteine residue at the ATP binding position. These tyrosine kinases include BLK, BMX, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), HER4, ITK, Janus kinase 3 (JAK3), TEC and TXK[117,130,131]. For instance, the prevalence of bleeding is up

to 44% and it is known that BTK, along with other members of the TFKs, are involved in platelet aggregation through glycoprotein VI signaling[132]. Therefore, the inhibition of on-target of BTK and off-target of other TFKs by ibrutinib is the potential mechanism for bleeding events occurred in patients.

In addition, diarrhea is the most prevalent ibrutinib-related side effect with 50% occurrence in patients[128,133–135]. The suggested mechanism of ibrutinib-related diarrhea is off-target binding to EGFR, since it is known that diarrhea is an adverse effect observed with EGFR inhibitors[136].

Moreover, ibrutinib could also affect other proteins without cysteine residue, like PTK6/BRK, C-terminal Src Kinase (CSK), FRG, hematopoietic cell kinase (HCK) and lymphocyte-specific protein tyrosine kinase (LCK), which might influence the efficacy or induce adverse effects of ibrutinib[118,131]. Therefore, toxicity is a common issue related with long-term treatment of ibrutinib, thus it is the main reason for discontinuous therapy of this compound.

1.3.2.1.2 Affect malignant B-cells and tumor microenvironment (TME) cells

In tissues, like lymph node or bone marrow, CLL cells proliferate at different microanatomical sites which are called proliferation centers or pseudo follicles[137,138]. CLL cells are dependent on external signals from the TME for their survival and evolution. CLL cells normally experience spontaneous apoptosis when they are cultured *in vitro*. Tissue stromal cells, for instance bone marrow stromal cells (BMSC) could prevent spontaneous and drug-induced CLL cell apoptosis in a contact-dependent manner[139,140]. The interactions between stromal cells and CLL cells occur both in bone marrow and in secondary lymphatic compartments in CLL patients[141]. In lymph nodes, CLL cells interact with various kinds of stromal cells, like $CD4^+$ T cells and $CD68^+$ nurse like cells (NLCs), which could also protect CLL cells from apoptosis[142–145]. Stromal cells constitutively secret chemokines and also transduce additional signals, which regulate CLL cell tissue homing, survival and proliferation[138]. Conversely, stromal cells are also activated by CLL cells via inducing PKC-β2 expression and subsequently activating NF-κB pathway[146]. In addition, NLCs, which are found in lymphoid tissues from CLL patients, can secrete chemokines like CXCL12 and CXCL13 to attract and protect CLL cells[142,147,148]. The interaction between NLCs and CLL cells via the expression of a proliferation-inducing ligand (APRIL) and the TNF family members B-cell activating factor (BAFF) on NLCs, protects CLL cells from apoptosis[149]. In CLL animal model, it has been demonstrated that NLCs can promote CLL disease progression[150,151].

In TME, CLL cells affect the composition and function of T-cells and NK cells in order to escape from immune-mediated cytotoxicity[152]. In CLL patients, the expression of inhibitory receptors such as programmed-death-receptor 1(PD-1) was found to be upregulated on T cells

and the number of $CD4^+CD25^{high}$ T regulatory cells (Treg) was increased in the TME[152]. These alterations might facilitate the evasion from anti-tumor immunity. Moreover, the phenotype of $CD8^+$ cytotoxic T-cells was also changed and formed defective immune synapses because both granzyme B packaging and degranulation have been impaired[153]. In CLL patients, both the response to soluble BCL2 associated athanogene 6 (BAG6) ligand secreted by CLL cells and the activating receptor NKp30 on NK cells were reduced in CLL patients, which restricted the NK cell–mediated antitumor activity[154,155]. CLL cells and their TME are depicted in Figure 4[156].

In CLL patients, the absolute lymphocyte count (ALC) increased temporarily after ibrutinib treatment, which is so-called "redistribution lymphocytosis". The ALC reduced rapidly during the off-drug period but went up again when patients restarted to take ibrutinib[110]. This observation, similar to all compounds targeting the BCR signaling, is due to the migration of lymphocytes from nodal tissues. And this migration is probably because the signaling disruption that affects adhesion factors in LNs and bone marrow, causes cell mobilization[157].

As discussed before, upon BCR engagement, BTK is rapidly activated by LYN and SYK, leading to the activation of downstream signaling which regulates B-cell proliferation and differentiation. Therefore, block of BTK activity by ibrutinib inhibits CLL cell survival and proliferation. In addition to BCR signaling, BTK is also implicated in signaling pathway of other receptors such as adhesion molecules (integrins) and CXCR4 and CXCR5 chemokine receptors, which are associated with B-lymphocyte adhesion and migration as shown in Figure 4[156,158]. In CLL patients receiving ibrutinib therapy, the drug also inhibits integrin-mediated adhesion and chemotaxis towards CXCL12 and CXCL13[159,160]. Interestingly, Ponader, S., *et al* found that the secretion of CCL3 and CCL4, being BCR activation-dependent chemokines in plasma, was downregulated both *in vitro* and *in vivo* from CLL patients receiving ibrutinib treatment[159]. In conclusion, ibrutinib inhibits CLL cell proliferation, migration and homing by affecting both CLL cells and the TME cells.

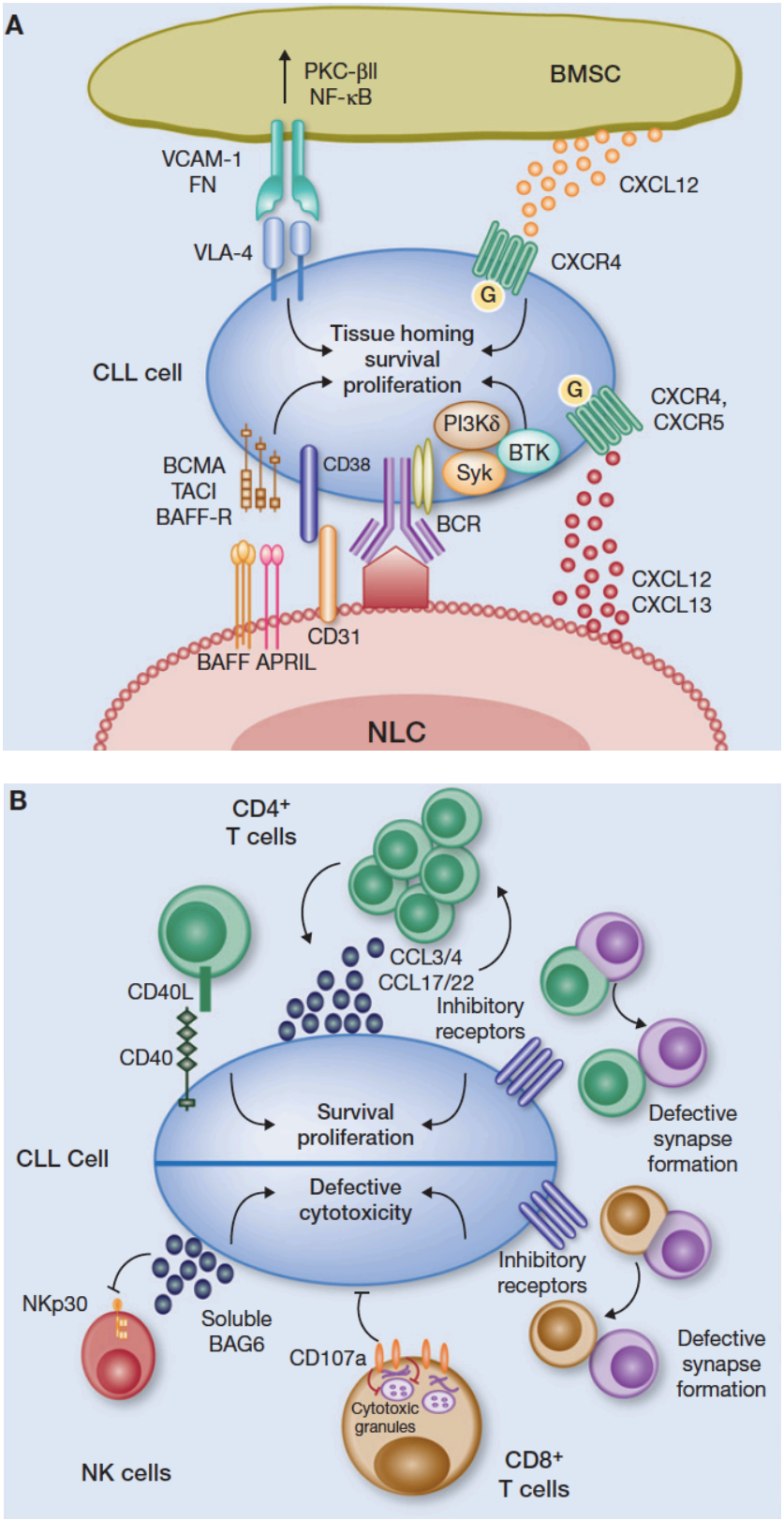

Figure 4. The CLL microenvironment. (A) The interaction between CLL cells and bone marrow stromal cells (BMSC) and nurse like cells (NLCs). (B) CLL cells and their interaction with T-cells and NK cells, which support CLL cell survival and proliferation. Adapted from ten Hacken E, *et al.* Clin Cancer Res. 2014. Reprinted with permission from publishers.

1.3.2.1.3 Resistance mechanisms for ibrutinib

The malignant cells developing resistance to the targeted treatments is the principal issue that restricts the efficacy of the therapy. Although ibrutinib shows good effectiveness in several B-cell malignancies, there are still a substantial group of patients who do not respond to it and some patients treated with ibrutinib develop resistance. Different ibrutinib resistance mechanisms have been summarized in Figure 5[122,161–168].

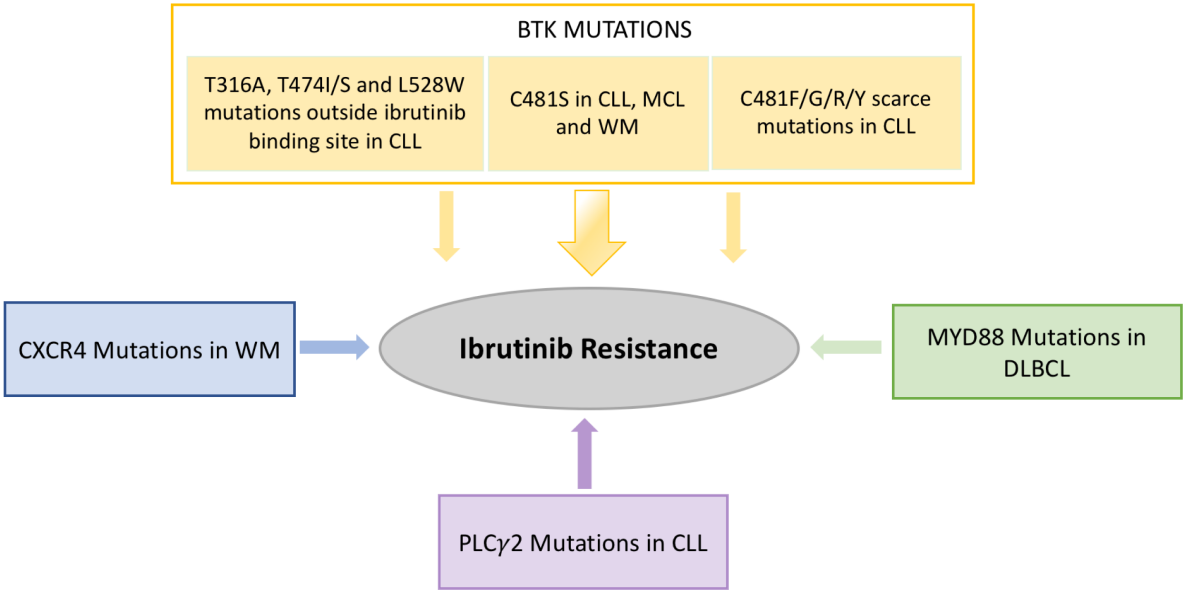

Figure 5. The summary of different ibrutinib resistance mechanisms in B-cell cancers.

Mutations of the molecules which are involved in the B-cell signaling pathway are the most frequent mechanisms of developing resistance to ibrutinib. Most mutations occur in BTK or its direct downstream molecule PLCγ2[169]. The most common mechanism of ibrutinib resistance in B-cell malignancies is cysteine-to-serine substitution at residue 481(C481S) in BTK, which accounts for more than 80% in resistant patients[162,170,171]. Within this substitution, the covalent binding of ibrutinib to BTK will be destroyed, which changes the irreversible inhibition to reversible inhibition and also reduces the binding affinity to BTK[161].

In addition to the BTK C481S mutation, various PLCγ2 mutations have also been reported to be involved in ibrutinib resistance in CLL patients. Woyach, J. A., *et al* found that in six ibrutinib-resistant CLL patients, three different PLCγ2 mutations were detected in two CLL patients, arginine-to-tryptophan mutation at site 665 (R665W), serine-to-tyrosine mutation at site 707 (S707Y) and leucine-to-phenylalanine at site 845 (L845F)[162,171]. Different from the BTK C481S mutation, which results in ultimate loss of BTK inhibition by ibrutinib, all the PLCγ2 mutations are gain-of-function mutations. These PLCγ2 mutations could induce the activation of BCR signaling constitutively and are not inhibited by ibrutinib. Some other BTK variations related with BTK C481S mutation or PLCγ2 mutations have also been found in few patients. These BTK mutations include T474I, T474S, C481F, C481G, C481R, C481Y and L528W, all of which occur in the catalytic kinase domain[122,172,173]. Moreover, a new mutation in the SH2 domain of BTK, threonine-to-alanine mutation at position 316

(T316A) has been identified in ibrutinib resistant CLL patients[174]. However, the resistance mechanism of this mutation is still unknown.

Currently, all these mutations occur before or during treatment is still on debate[175]. The most common view is that resistant BTK or PLCγ2 mutations exist at the baseline before drug exposure in ibrutinib-treated CLL. However, the numbers of mutant CLL sub-clones are too small to be detected. Notably, highly sensitive technologies (such as droplet microfluid technologies) have been developed and applied to facilitate detecting these mutations before ibrutinib treatment initiation. This supports the idea that external selection pressure coming from targeted treatment promotes the selection of rare sub-clones that are already existed prior to treatment[170].

1.3.2.2 Second generation BTK inhibitors

Even though ibrutinib shows ideal effectiveness in patients, due to the development of resistance and its off-target adverse effects, second generation of BTK inhibitors have been developed, which has improved binding specificity and maintained effects against mutated BTK variants [176,177]. Several other BTK inhibitors are currently being tried either singly or in combination, both in the frontline and the R/R conditions.

Acalabrutinib (ACP-196) is a new selective second generation BTK inhibitor, which has higher specificity than ibrutinib and it was approved by FDA for the treatment of MCL in 2018. Acalabrutinib seems to have less adverse effects than ibrutinib because of its much high specificity. This compound also binds to Cys481 covalently and irreversibly[176,177]. Even though acalabrutinib shows high overall response rate (ORR) in patients with R/R disease, whether this drug is more efficient than ibrutinib still needs a further follow-up[177]. There is an ongoing phase III study which compares acalabrutinib with ibrutinib.

Another BTK inhibitor, vecabrutinib (SNS-062) seems to have good efficacy and inhibits BTK activity *in vitro* in CLL cells harboring BTK C481S resistance mutation[178]. Several other BTK inhibitors such as CC-292 (Spebrutinib), BGB-3111 (Zanubrutinib), ONO/GS-4059 (Tirabrutinib) and so on are currently under evaluation for the treatment of lymphomas and autoimmune diseases. BTK inhibitors are summarized in Table 2.

Table 2. The summary of BTK inhibitors, which are tested in lymphomas and autoimmune diseases.

BINDING TYPE	BTK INHIBITORS	STATUS
COVALENT	Ibrutinib	CLL, MCL, MZL, WM, GVHD-FDA
	ACP-196 (Acalabrutinib)	MCL-FDA; CLL, WM, RA-Phase II
	CC-292 (Spebrutinib)	RA-Phase II; CLL-Phase I
	BGB-3111(Zanubrutinib)	MCL-FDA; CLL, WM-Phase III
	CT-1530	CLL, MCL-Phase I/II
	ONO/GS-4059 (Tirabrutinib)	CLL, WM-Phase II; RA-Phase I
	M7583	BCL-Phase I/II
	ICP-022 (Orelabrutinib)	WM-Phase II; CLL, MCL-Phase I/II; RA-Phase I
	TG-1701	CLL-Phase I
	ABBV-105	RA-Phase II
	TAS5315	RA-Phase II
	SHR1459	CLL-Phase I
NON-COVALENT	BMS-986142	RA-Phase II
	SNS-062 (Vecabrutinib)	CLL-Phase I/II
	DTRMWXHS-12	MCL-Phase I
	RN-486	CLL-Preclinical
	GDC-0853 (Fenebrutinib)	RA-Phase II

2 AIMS

The overall aim of this thesis is to address the effect of BTK activity inhibition and BTK mutations in different diseases. In CLL patients, BTK inhibitor, ibrutinib prevents tumor cell survival by disrupting mainly BCR signaling and also affects other cell populations which regulate cell migration and homing. Ibrutinib binds to the site of Cys481 in the BTK kinase domain, for which serine substitution is the most common resistance mechanism in patients. Whether other potential amino acid replacement at C481 by nucleotide changes would cause resistance is unknown. Moreover, LOF mutations of BTK cause XLA in males and the mutation spectrum is still under investigation. The individual aims related to each paper are listed as following.

Paper I

• To identify other BTK mutations at Cys481 that could induce ibrutinib resistance.

• To characterize all the possible BTK mutations at the ibrutinib binding site.

Paper II

• To investigate the change of inflammation biomarkers in plasma in CLL patients with ibrutinib therapy.

• To characterize the early effect of ibrutinib in patients in various cell populations in lymph node and peripheral blood.

• To explore the dynamics of ibrutinib-induced transcriptional alterations in CLL patients.

Paper III

• To make predictions on the tolerance to amino acid replacements in BTK.

• To validate several unreported BTK variants in a cellular context by assaying their catalytic activity.

• To investigate BTK mutations in three groups of malignancies, BTK-dependent tumors, BTK-independent tumors and BTK-potentially-dependent tumors.

3 MATERIALS AND METHODS

The following chapters briefly describe some relevant methods used in this thesis. More details about the methods can be found in the respective papers.

3.1 CELL SOURCES

In paper I, to investigate the stability and catalytic activity of different BTK variants, several cell lines were used. The cell lines include non-lymphoid cell lines such as COS-7 (African green monkey fibroblast-like kidney) and HEK-293T (human embryonic kidney cells), as well as lymphoma origin cells, DT40 (chicken lymphoma cells). Two out of the three cell lines were purchased from the American Type Culture Collection. The DT40 with inactivated BTK cell line, B7.10 was generated by Dr T. Kurosaki's laboratory, Japan, and generously provided to us[179]. In paper II, all the experiments were performed in primary cells sorted from patient samples. In paper III, COS-7 and HEK-293T cell lines were used to assess the catalytical activity of several BTK variants.

3.2 PLASMID TRANSFECTION

In both paper I and paper III, plasmids with respective BTK mutations were transfected into cell lines. Two transfection techniques were performed in this thesis depending on the characteristics of cell lines. For adherent cells, COS-7 and HEK-293T cells, plasmids were transfected by using polyethylenimine (PEI) (Polyscience, Inc., Warrington, PA, USA). Whereas, for suspension cell type, B7.10 cells, we used electroporation to transfect plasmids by the Neon transfection system according to the manufacture's protocol (Life technologies, La Jolla, CA, USA).

3.3 PROTEIN ANALYSIS

3.3.1 Western blot (WB)

In both paper I and III, we analyzed the BTK expression and activity of different variants by WB and compared it with wild-type BTK. Protein was extracted from transfected cells and mixed with sample buffer (0.4M sodium carbonate, 0.5M ditiothreitol, 8% SDS and 10% glycerol) and then heated for 5 minutes at 65°C.The mixture was loaded onto a 4-12% Bis-Tris Protein gel and run at MES SDS running buffer at 120V for around 2 hrs. After this, the iBlot system was used to transfer the proteins on the gel to a nitrocellulose membrane. Membranes were incubated with blocking buffer for 1 hr at room temperature (RT) and followed by the incubation with primary antibody at 4°C overnight. The membranes were washed and then incubated with secondary antibodies for 45 minutes at RT. After this,

membranes were washed again and then scanned using the Odyssey infrared imaging system (Li-COR Biosciences GmbH).

3.3.2 Immunoprecipitation (IP)

In paper I, in order to obtain purified BTK and PLCγ2, IP was performed. IP is a common technique to isolate and purify proteins from heterogeneous protein mixtures. Cells were lysed in lysis buffer and then protein mixture was extracted. The protein mixture was incubated with the corresponding antibody that binds to the targeted protein. After incubation, the interacted antibody-protein mixture was pulled down by protein A/G beads to isolate the targeted protein. This method is also applied to identify protein-protein interactions.

3.4 *IN VITRO* KINASE ASSAY

In paper I, in order to demonstrate whether PLCγ2 was the direct substrate of BTK, *in vitro* kinase assay was performed. This technique can detect the catalytic activity of a kinase in a purified form instead of in whole cell lysates. BTK and PLCγ2 were purified and isolated by IP firstly. Subsequently, wild-type BTK or BTK variant protein was incubated with PLCγ2 and ATP in the kinase reaction buffer for 30 minutes at 30°C. This specific buffer enables BTK to transfer a phosphate group (the gamma-PO4) from ATP to PLCγ2. The reactions were suspended by adding sample buffer and followed by WB analysis.

3.5 PATIENT SAMPLE ANALYSIS

3.5.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation

In paper II, peripheral blood (PB) and lymph node (LN) samples from CLL patients were collected before treatment and at a series of timepoints after treatment initiation. Firstly, PB samples were spun down at 500g for 5 mins to obtain plasma, which was frozen at -80°C for later analysis. The left blood was mixed with DPBS and followed by the density gradient centrifugation using Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden) to isolate PBMCs.

3.5.2 Flow Cytometry

Flow cytometry is a routinely used technique to detect physical and chemical characteristics of a population of cells. By incubating with specific antibody mixture, different populations of cells could be sorted out separately. This technique is one of the main methods used throughout the paper II.

Mononuclear cells were first blocked with FcR and then stained for 30 minutes at 4°C with a group of antibodies. Subsequently, propidium iodide (PI) (Life Technologies, Carlsbad, CA) was added to exclude dead cells. Cell analysis and sorting was performed on a FACS ARIA III or Fusion and data was analyzed using FlowJo v9.9. CLL cells were purified and sorted as the population with PI-CD11b/CD14/CD16/CD56^{-}CD3^{-}CD19^{+}CD5^{+}. Depending on the characteristics of different cell populations, normal B-cells, T-cells, NK cells and dendritic cells (DCs) were also sorted and analyzed.

3.5.3 Proximity Extension Assay (PEA)

Proximity Extension Assay (PEA) is a high throughput immunoassay for the detection of protein biomarkers in liquid samples. This method is performed by using 96x96 format (Olink Bioscience, Uppsala, Sweden)[180]. For each biomarker, unique oligonucleotides that used as probes are linked to a matched pair of antibodies, which bind to the respective targeted proteins. Subsequently, the probes hybridize and bind to each other. DNA polymerase is then added to promote an extension of the hybridizing oligo to obtain a DNA amplicon.

In paper II, we used PEA to measure the biomarkers in plasma samples. Ninety-two inflammation-related biomarkers were investigated simultaneously. For each sample, 1 μl of plasma was taken for each measurement and then duplicate measurements were run. Statistical analysis was performed to compare levels of the biomarkers at various timepoints during treatment with the levels at baseline.

3.6 IDENTIFICATION OF NOVEL BTK VARIANTS IN XLA PATIENTS

In paper III, we studied a large cohort of XLA patients and identified BTK mutations in these patient samples. 108 unrelated patients were collected and analyzed in Karolinska Institutet (KI) in Sweden. All the patients were males and diagnosed as carrying XLA. The diagnosis was made based on low levels of all isotypes of Igs, the very low levels or even absence of circulating B cells, and susceptibility to infections. Sanger sequencing was performed to detect the variations. The detailed information can be found in the previous publication[44].

4 RESULTS AND DISCUSSION

4.1 PAPER I

Ibrutinib is the first approved BTK inhibitor, which shows high efficacy in CLL patients and is also the first BTK inhibitor to which patients have developed resistance. The most common resistance mechanism is cysteine-to-serine mutation at C481 site of BTK.

In this study, we generated all the six possible BTK variants at Cys481 site and we also included one threonine variant since serine has similar function and structure with threonine, even though two nucleotide changes are required. Plasmids encoding BTK substitutions with C481F, C481G, C481R, C481S, C481T, C481W and C481Y were transfected into a non-lymphoid cell line and all the variants were demonstrated to express equally and stably.

In order to explore the relevance between the structure and the function of the variants, plasmids were transfected to both non-lymphoid cell line and BTK-deficient chicken B cells. The phosphorylation status at Y223 and Y551 of BTK and the catalytic activity of all the seven BTK variants were analyzed. We found that variants with either serine or threonine substitution retained catalytic activity, whereas the glycine variant displayed enzymatic activity but to a much lesser extent than serine or threonine replacement. Conversely, the phosphorylation of other four BTK variants was compromised severely and these variants could not phosphorylate PLCγ2, which is a direct BTK substrate.

C481 is a functionally essential site implicated in ATP binding and in the constitution of the catalytic conformation of BTK. The majority of amino acid substitutions at this position are predicted to be pathogenic both by PON-BTK and PON-P2 tolerance predictors[44,181]. In the BTK active conformation, C481 involves in the orientation of the catalytic R525. Since serine and threonine replacements can still form similar interactions as the original cysteine, these two BTK variants could be functional. For the variants with C481F, C481W and C481Y, the aromatic side chains of these amino acids are big and bulky, which results in structural conflicts within the kinase domain and the substrate. For the C481R variant, the arginine side chain is long and flexible, and while it can be accommodated to the structure, it is unable to form the catalytic conformation of BTK because of the charged side chain. However, BTK C481G variant showed activity since it does not contain any side chain and then the binding could be still allowed. This variant seems to have the right conformation but does not have functionally important side-chain interactions.

To investigate the sensitivity of the active BTK variants to ibrutinib, the cell lines mentioned above were transfected with plasmids encoding BTK C481S or C481T variant and then treated with sequential concentrations of ibrutinib. In wild-type BTK, the activity of BTK was inhibited strongly with 0.5 μM ibrutinib, whereas the two active variants required much higher concentration of ibrutinib to block their activity. Moreover, ibrutinib washout experiments were performed and demonstrated that the activity of C481S and C481T variants could only be inhibited when they were incubated with high concentration of ibrutinib

continuously. Therefore, we concluded that in addition to C481S variant, the C481T replacement could also be one potential ibrutinib resistance mechanism. However, since the C481T variant needs two nucleotide changes, its occurrence is expected to be much lower than C481S variant.

In addition, we also performed the modeling of the BTK kinase domain structure with ibrutinib. As described above, serine and threonine can maintain similar interactions as cysteine, thereby both variants are functional. They have an -OH end group instead of the -SH group and contain electrostatic properties and hence form interactions. However, serine and threonine are incapable of forming covalent bonds with ibrutinib, which make them insensitive to ibrutinib. The C481G variant is unable to activate downstream signaling fully, and therefore it is not expected to be crucial for tumor cell survival during ibrutinib treatment. For those inactive BTK variants, we propose that they would hamper tumor cell survival since active BTK is essential for the growth of B-cell derived malignant cells. The ibrutinib sensitivity of all the variants and their effects on downstream signaling are summarized in the following figure 6.

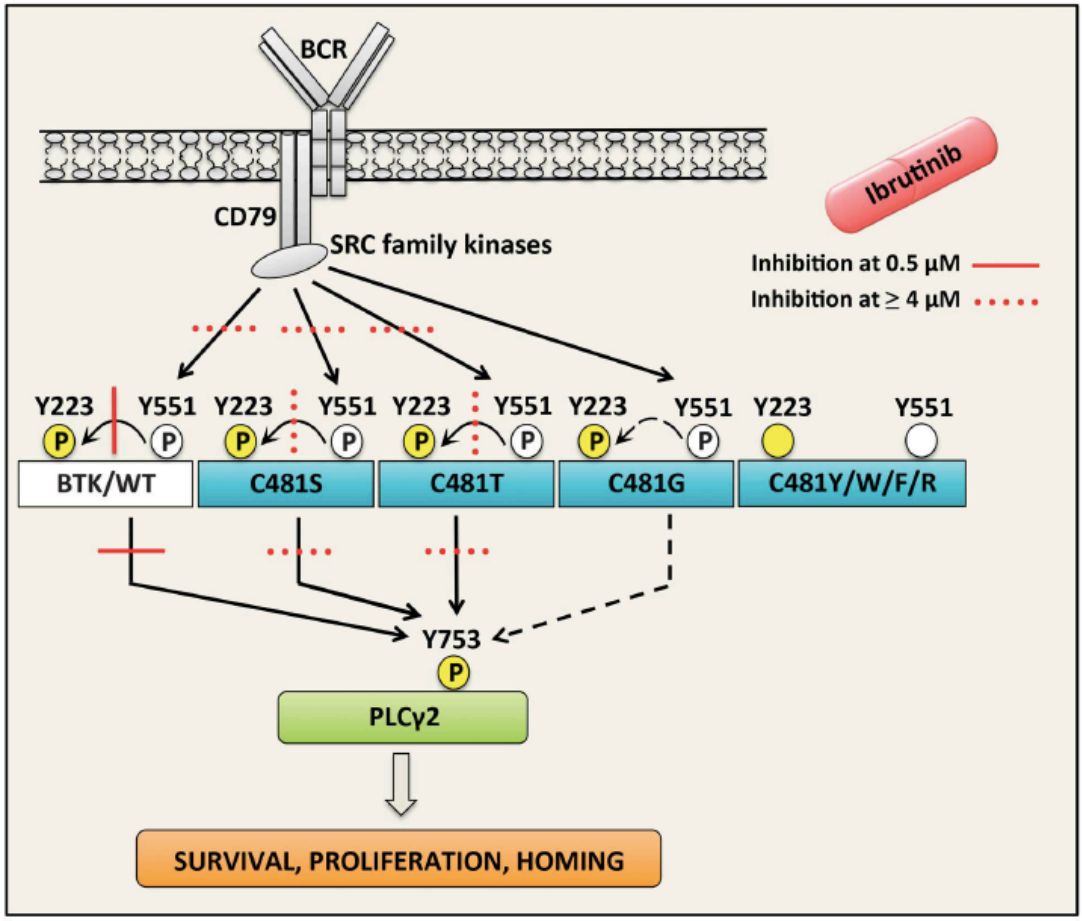

Figure 6. Schematic diagram of the BCR signaling pathway displays the sensitivity of BTK C481 variants to ibrutinib and their effects on downstream signaling[182].

4.2 PAPER II

As discussed previously, in CLL patients, ibrutinib is the first FDA-approved BTK inhibitor for therapy. Many patients with ibrutinib treatment have described that they had an nearly immediate sensation of improved well-being after the first dose of this drug. However, the reason for this clinical phenomenon is unclear. In this study, we characterized thoroughly of ibrutinib-induced changes at inflammatory, transcriptional and cellular aspects in both LN and PB samples immediately after the start of the treatment. We have compared the changes before treatment and after treatment in LN and PB, as well as the difference between LN and PB.

We collected LN and PB samples before treatment and at 6 time points after treatment initiation, which are 9h, days 2, 4, 8, 15 and 29. Firstly, we assessed the inflammation-related biomarkers in plasma samples. Plasma was collected from PB and the levels of 92 inflammation-related protein biomarkers was analyzed at pre-treatment and at 6 time points during the treatment. We found that the levels of 23 molecules were down-regulated more than one time points. To explore if the changes corresponded to the mRNA levels, we performed RNA-sequencing in CLL cells sorted from LN and PB. In plasma samples, the expression of CCL2, CCL3, CCL4, CCL19, IL-10, TGF-α and TNF-β was already decreased by 9h and continuously down-regulated throughout all the following time points. CCL3 and CCL4 are considered to be prognostic markers in CLL and our RNA-sequencing data propose that these two chemokines may mainly originate from CLL cells resident in LN. Most of the 23 molecules were found to be not expressed in sorted CLL cells, which suggested that ibrutinib might influence directly other BTK-expressed cells instead of B cells or CLL cells. The effect could also be on e.g., other kinases in these cells. For example, the downregulation of CCL2 is probably because ibrutinib affects monocytes, which are the main cell source of CCL2 production. In addition, the majority of the downregulated cytokines are pro-inflammatory, which is consistent with previous study demonstrating that inflammatory cytokines might prevent CLL cells from apoptosis both *in vivo* and *in vitro* [183].

We also performed RNA-sequencing on the LN and/or PB samples before treatment, at day 2 and day 29 to explore the entire effect of ibrutinib on RNA level. The levels of 357 genes was changed at day 2 after treatment initiation in CLL cells from both LN and PB. Next we looked into the function of the affected genes, and it showed that the upregulated genes were related to ribosome assembly and translation. Whereas, the genes downregulated were linked to B-cell proliferation, hematopoiesis, leukocyte cell-cell adhesion and regulation of kinase activity. In addition, the downstream molecules of BCR and NF-κB signaling, E2F and MYC target genes have been previously reported to be highly expressed in LN CCL cells from CLL patient[184]. The similar scenario occurred in our study, in which the levels of BCR, NF-κB and E2F target genes were significantly higher in LN CLL cells than in PB CLL cells. Ibrutinib treatment induced significantly decrease of BCR, NF-κB, E2F and MYC target genes in LN. However, in PB, only the BCR downstream genes showed continuous reduction after

treatment, while E2F and MYC target genes were only downregulated at day 2 after ibrutinib treatment.

It is a common observation that ibrutinib treatment induces a rapid increase of ALC in CLL patients, which is called 'redistribution lymphocytosis'. This phenomenon is due to the migration of lymphocytes from LN to PB after ibrutinib treatment, which is probably because ibrutinib affects some adhesion factors in LN[157]. We observed the same phenomenon that ALC was significantly increased at days 2, 8 and 15. In four CLL patients, the ALC decreased at day 29 probably because CLL cells without supported TME like LN went to apoptosis. However, circulating T-cells were not affected while the proliferating T-cells were reduced significantly at day 29.

In addition, we assessed the changes of cell activation and migration markers between paired LN and PB samples during treatment. Only the expression of CD23, which is also a diagnostic marker in CLL, was found to be higher in LN than PB at baseline[185]. CD23 expression was reduced from day 2 in LN, but was downregulated much later in PB, at day 29. In healthy individuals, the interaction between $CD23^+$ immature B-cells and T-helper cells could stimulate BCR signaling, which might cause autoimmunity[186,187]. After ibrutinib treatment, the early reduction of CD23 level in LN suggests that CD23 may be the most BCR-dependent CLL surface antigen as well as a good marker for the downregulation of BCR signaling. CD5, as a CLL marker, was highly expressed in LN CLL cells but was found to be downregulated only in CLL cells in PB at day 29. This may be because CD5 induces autoreactive B-lymphocyte tolerance, thereby restraining BCR activation and preventing cell expansion[188,189].

In CLL cells, the surface markers such as CXCR4 and CD49d are highly expressed, being important regulators for cell trafficking. In our study, both CXCR4 and CD49d were not decreased in PB and LN CLL cells. The interaction between CXCR4 and CXCL12 was suggested to be the mediator for CLL cell homing[190]. Our data indicated that the migration of CLL cells from LN to PB may be independent of CXCR4 downregulation in these patients.

At day 29, we also observed the decrease of $CD16^+SLAN^+$ monocytes. This type of monocytes can secrete high levels of TNF-α. Consistent with the low SLAN expression in an XLA patient, which lacks functional BTK, our results suggest that the reduction of $CD16^+SLAN^+$ monocytes was associated with ibrutinib therapy. CLL cells or T-cells in TME also can secrete TNF-α to inhibit plasmacytoid dendritic cells (pDCs) development[191]. In our patients, no pDCs were detected in three of five patients and pDCs population was upregulated during treatment, which could be attributed to the reduction of TNF-α produced by CLL cells or T-cells in TME upon ibrutinib treatment.

In conclusion, we demonstrated that ibrutinib treatment leads to a rapid interruption of an ongoing inflammatory response and impedes diverse pathways not only in CLL cells but also in TME, which mainly occur in LN. Moreover, we confirmed the association between BTK and chemokine synthesis, which might facilitate the development of therapy in future.

4.3 PAPER III

BTK is an essential component in B-cell development and is important for the B-cell dependent tumor progression. Loss of function mutations in BTK cause the primary immunodeficiency disease, XLA. Therefore, BTK acts as an activator in both B-cell development and progression of BTK-dependent tumors.

In this study, firstly we analyzed a large cohort of XLA patients and compared the mutation spectrum with those occurring in leukemia and lymphoma in order to investigate the role of BTK in different malignancies. We focused on amino acid substitutions of BTK in this project, in which three groups of malignancies, BTK-dependent, BTK-potentially-dependent and BTK-independent, were dissected. As mentioned in the previous chapters, BTK inhibitors have been FDA-approved to treat B-cell malignancies, such as CLL, MCL and MZL. Based on the published information, we found that no XLA-causing mutation was observed in a big number of CLL (n=1,115) and MCL (n=239), which is consistent with our hypothesis that these tumors are sensitive to BTK inhibitors so they should not have XLA-type mutations[167,172,192–197].

Moreover, we studied amino acid substitutions in a large group of non-B-cell tumors from a mutation database[198]. There are 161 patients reported with amino acid replacements from 10,086 patients. This data is used as a comparison in our study.

The third group of tumors, are those hypothesized to be BTK-potentially-dependent, like DLBCL, FL and GC B-cell derived lymphomas. Since a subgroup of these tumors is sensitive to BTK inhibitors, XLA-type mutations should be selected against. Previously, it was proposed that BTK acted as a tumor suppressor by stabilizing p53 expression *in vitro*. Additional study displayed that lack of BTK expression promoted tumor formation in BLNK/SLP-65 deficient mice[70]. From our analysis, we found that the number of XLA-type mutations (the sum of known + predicted) was significantly higher than the number of such mutations in the non-B-cell tumors both if we considered the full-length BTK or only the kinase domain ($P \leq 0.0005$)[195,198–201], which supported the hypothesis about the role of BTK as a tumor suppressor in BTK-potentially-dependent malignancy.

In the large cohort of XLA patients analyzed in our study, six new amino acid substitutions: S38P, Y39H, F98L, V306D, R332L and H333L, of which R332L and H333L occurred in the same patient, were identified. Among them, three are new affected sites, S38, V306 and R332 where no XLA mutations have been reported to date. Concerning the patient containing two mutations, R332L and H333L, the tyrosine substitution at H333 site has been identified to cause XLA, whereas the role of R332L is not clear. However, the SH2 domain recognizes pY-containing sequences as described previously and H333 was found to be involved in binding the pY-containing proteins. Therefore mutations at this site have high potential to cause disease[202,203].

We have also performed predictions of the tolerance to every single amino acid replacement for the full-length BTK. In general, amino acid substitutions distribute throughout the whole BTK, except the SH3 domain where only two substitutions have been reported so far. In addition, there are some special regions without known XLA-causing variants or with several XLA variants. We chose three such sites, Q467, E599 and R618 in the kinase domain and generated a group of novel amino acid substitutions. Variants at these positions, which were predicted to be either neutral or pathogenic, were produced. The predicted neutral substitutions are Q467H, Q467L, E599K and R618K, and the predicted pathogenic replacements are Q467P, E599V and R618M. Their function was validated *in vitro* and the results were consistent with our predictions. For the variants predicted to be neutral, both BTK and PLCγ2 were phosphorylated, which reveals that these amino acid substitutions indeed are tolerated.

For the whole BTK, the solvent accessibility of each residue was explored and all amino acids were assigned to be exposed, buried or intermediate. We have made calculations about the tolerance of amino acid substitutions for each domain and we found that tolerance at buried positions is lower than exposed positions in the KD, which was the same as previously reported[44]. Similar scenario occurred in the PH domain, whereas not so obvious in SH2 domain, since many exposed sites which are involved in the BTK structural formation, are highly mutated. Few mutation sites have been identified in the TH and SH3 domains so that we could not make any conclusion.

In addition, amino acid replacements seem to be less tolerated in secondary structural elements, including helices and β-strands, than in loop regions. We have integrated the secondary structures of the whole BTK and investigated the substitution tolerance. Generally, amino acid substitutions occurred in higher frequency among the residues belonging to the helices (36.7%) and followed by β-strands (28.8%) as compared to the loop regions (24.2%). However, this phenomenon is not the same if we consider every single domain, suggesting caution for the evaluation of rare mutation sites, like the TH and SH3 domains, as discussed above. For malignancies, we observed that mutations are also highly tolerated in loop regions especially for the BTK-potentially-dependent cancers.

In conclusion, we have studied the function of BTK in malignancies, and it seems that BTK acts as a tumor suppressor in the BTK-potentially-dependent cancers. To the best of our knowledge, in patients with hematopoietic malignancies, there has never been any clear evidence for the function of BTK as a tumor suppressor and this was addressed in this report.

5 CONCLUSIONS

BTK is a crucial component in the BCR signaling pathway, which regulates B-cell survival, proliferation and differentiation. BTK mutations can be XLA-causing or be neutral, which depends on the alterations of the protein stability and function. For malignancies, BTK is activated in several lymphoma and leukemia cells. Therefore, BTK inhibitors, especially ibrutinib, have been developed rapidly and show high efficacy in patients. However, certain number of patients develop resistance to ibrutinib and the main mechanism is the cysteine-to-serine mutation at C481 site of BTK, to which ibrutinib binds. In the paper I, we demonstrated another potential resistance mechanism, which is the cysteine-to-threonine mutation at C481 position. In paper II, the very early effects of ibrutinib in CLL patients have been investigated. Our data illustrated that ibrutinib affected the expression of several chemokines as early as 9h after treatment initiation. In addition, RNA-sequencing results indicated that ibrutinib significantly downregulated the levels of BCR, NF-κB and E2F target genes, which are associated with CLL cell survival and proliferation. Moreover, ibrutinib affected other cell populations, such as $CD16^{+}SLAN^{+}$monocytes. Therefore, the improved well-being of CLL patients treated with ibrutinib is due to the comprehensive effects of ibrutinib not only on B-cells but also on other cell populations and chemokine or cytokine synthesis. In paper III, we have compared the germline mutations of a large number of XLA patients with the acquired mutations in BTK-potentially-dependent tumors. The data suggests that BTK acts as a potential tumor suppressor in a subset of this group of malignancies. The conclusions of the three papers are summarized as shown in Figure 7.

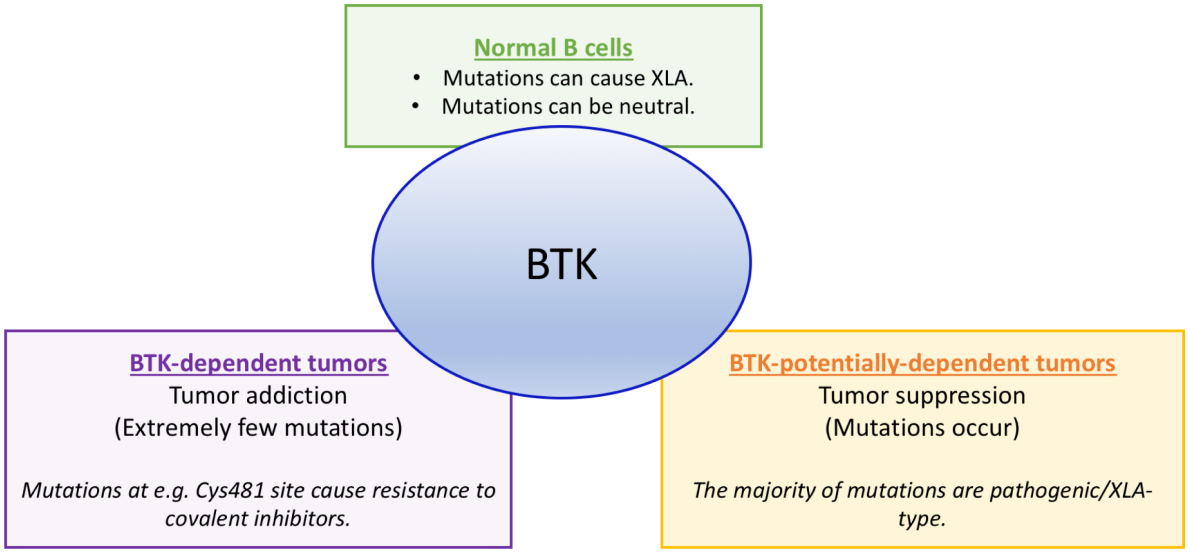

Figure 7. Schematic representation of summarizing the three papers.

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